ALIMENTARY TRACT

Composition of gastro-oesophageal refluxate

D C Gotley, A P Morgan, D Ball, R W Owen, M J Cooper

Abstract

Fifty two patients with abnormal acid gastro-oesophageal reflux were studied by simultaneous oesophageal pH monitoring and continuous aspiration for 16 hours. Aspirates (from discrete two hour periods) were analysed for volume, pH, bile acids (conjugated and unconjugated), trypsin, and pepsin. The results were compared with pH changes and degree of oesophagitis. Patients with oesophagitis had greater acid reflux than those without, but patients with stricture and Barrett's oesophagus had similar acid reflux to those with uncomplicated erosive oesophagitis. Pepsin concentrations were highest in patients with stricture and Barrett's oesophagus particularly during nocturnal periods. Conjugated bile acids were detected in 75% of patients, mainly during the night, but only 2% of aspirates contained concentrations likely to be cytotoxic. Unconjugated bile acids were not detected, and trypsin was seldom found. Reflux oesophagitis is caused by acid and pepsin. Bile acids and trypsin are probably unimportant.

Reflux oesophagitis is caused by excessive exposure of the oesophageal mucosa to refluxed gastric contents,¹ but the relative importance of each component of the gastric juice is unknown. Although acid has been implicated,² intra-oesophageal acid exposure does not correlate accurately with the degree of oesophagitis,^{3,4} and suppression of gastric acid secretion does not always heal oesophageal erosions.⁵⁻⁷ Pepsin, which causes severe oesophageal erosions in an acid medium,⁸ and duodenal contents (such as bile acid and trypsin) are also thought to be important in the pathogenesis of reflux oesophagitis.⁹⁻¹¹ The aim of this study was to determine the exact composition of refluxate in patients with gastro-oesophageal reflux, its relation to intraoesophageal pH changes, and the degree of mucosal damage.

Methods

PATIENTS

Fifty two patients (37 men, 15 women), median age 56 years (range 24-85 years), with chronic (>6 months) symptomatic gastro-oesophageal reflux were studied. Thirty four (65%) had not responded to medical treatment, although no

patient had been given omeprazole. No patient had had previous oesophageal or gastric surgery. All patients stopped taking drugs likely to affect gut motility or secretion 48 hours before investigation and each gave informed, written consent.

CONTROL SUBJECTS

Twenty asymptomatic control subjects (12 men, eight women), median age 46 years (range 21-72 years), were studied by oesophageal pH monitoring. None had had symptomatic gastro-oesophageal reflux or previous gastrointestinal surgery. None underwent endoscopy.

SYMPTOM SCORE

Patients were interviewed and symptoms of reflux were graded using an established system.¹² A score from 0 (absent) to 3 (severe) was allocated for heartburn, regurgitation and dysphagia.

ENDOSCOPY

Four groups were identified on the basis of endoscopic findings: normal oesophagus (grades 0-I; n=16), erosive oesophagitis (grades II and III; n=17), benign stricture (grade IV; n=11), and Barrett's oesophagus (n=8) (Table I). A stricture was defined as a stenosis which prevented the passage of a 10 mm diameter GIF XQ20 endoscope (Olympus, Keymed, Southend, UK) irrespective of whether there was histological evidence of metaplastic epithelium below the stricture. The criteria for Barrett's oesophagus were >3 cm of metaplastic epithelium lining the tubular oesophagus, confirmed histologically. Patients with Barrett's oesophagus and early ('soft') stricture formation which did not hinder the passage of the endoscope (n=2) were allocated to the Barrett's group. Erythema of the mucosa without ulceration was classified as 'normal'.¹² Patients with gastric or duodenal ulceration were excluded from the study. Those with a stricture underwent oesophageal dilatation before further study.

TABLE I Patient groups according to endoscopic findings

	Grade of oesophagitis			
	0-I	II-III	IV	Barrett's
No of patients	16	17	11	8
Median age (range) (years)	43 (24-81)	55 (30-80)	73 (32-85)	48 (25-84)
M:F	12:4	12:5	7:4	6:2

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PRELIMINARY 24 HOUR pH MONITORING STUDY

Preliminary ambulatory 24 hour intraoesophageal pH monitoring was carried out in patients and controls. An antimony pH electrode (calibrated in buffers of pH 7.01 and 1.07) was positioned 5 cm above the lower oesophageal sphincter (determined manometrically) and pH was recorded at 0.25 Hz using a portable digital recorder (Synectics, Sweden). An episode of acid gastro-oesophageal reflux began when the pH fell below 4 and ended when the pH rose above 5. Data were analysed for the number of reflux episodes, the number of episodes >5 minutes' duration, and the longest reflux episode. Acid exposure was expressed as the percentage of study time in which the pH was <4 (reflux fraction), and mean time (in minutes) per reflux episode (oesophageal clearance). All parameters were determined separately for upright, supine, and postprandial periods. Acid reflux was considered abnormal when the reflux fraction was >2 standard deviations higher than control fraction – that is, >4.5%.

COMBINED OESOPHAGEAL ASPIRATION AND pH MONITORING

After a six hour fast, a size 14 FG double lumen Salem sump tube with an attached antimony pH electrode was passed transnasally and positioned with its tip 5 cm above the lower oesophageal sphincter. After one hour (to minimise intubation effects) continuous oesophageal aspiration was started using suction apparatus connected to the air vent (inner lumen) of the sump tube. Simultaneous pH monitoring was maintained as above. Measurements started at 1700 hours and were stopped at 0900 the following morning. A standard meal, providing 20 g protein, 20 g fat, 83 g carbohydrate (595 kcal), was given at 1900, and aspiration was stopped for 30 minutes during its ingestion. Patients retired to bed between 2300 and 0700 and were allowed their

usual number of pillows; otherwise they sat upright in a chair. Daytime fasting (1700–1900), postprandial (1930–2100), and nocturnal (2300–0700) periods were assessed individually. Aspirates were collected in a flask over ice in eight two hourly aliquots (1½ hours for postprandial specimens), separated from mucus with a 0.2 mm wire mesh, centrifuged at 3000 rpm, and decanted. Specimens were stored at –70°C; those for trypsin analysis were titrated to pH 7.0 before storage to inhibit peptic activity. Specimens for pepsin assay were stored at a pH range within 1.8–6.0, which preserves maximal peptic activity.^{13,14} pH traces were analysed for reflux fraction and oesophageal clearance as described above. Both the 24 hour pH monitoring and combined aspiration/pH monitoring studies were carried out within four weeks of endoscopy.

BIOCHEMICAL ANALYSIS

After thawing, pH (measured using a combined glass electrode, Radiometer, Copenhagen) and volume were recorded. Specimens were analysed for pepsin, conjugated and unconjugated bile acids, and trypsin.

Pepsin

Total peptic activity was measured using a haemoglobin substrate assay.¹⁵ Aspirates were diluted with 0.01 mol/l HCl (1:10 vv) and incubated at 37°C for 15 minutes with denatured human haemoglobin. The digestion was terminated with 0.3 mol/l trichloroacetic acid. Absorbance of the supernatant, measured at 280 nm, was related to a series of standard solutions of pepsin prepared from hog gastric mucosa (2200 IU/mg).

Conjugated bile acids

Conjugated bile acids were measured using a modification of the technique of reversed phase ion suppression/ion paired high performance liquid chromatography.¹⁶ Bile acids were extracted with methanol (1:1 vv), centrifuged at 3000 rpm, and passed through a 0.22 µm Millex filter (Micropore, Molsheim, France), and 20 µl was injected onto the chromatograph (Gilson 302, model 71-25, USA). The mobile phase consisted of acetonitrile/water (54%) titrated to pH 2.5 with orthophosphoric acid. Tetrabutylammonium phosphate (0.4 mol/l) was used as the counter ion. The mobile phase flow rate was 0.5 ml/min at 1500 psi and ambient temperature (22–23°C). The stationary phase consisted of two columns in series (Altex Ultrasphere ODS 5 µm, USA; and Varian micropak SP C18-5, USA), and a Gilson Pri-column (SSI 05-0418) was used as a filter. The ultraviolet detector (Gilson, USA) was set at 210 nm with a range of 0.1 au, and elution time was 18 minutes per sample. Area under the peaks was calculated using an Apple IIe microprocessor controller/programmer. This assay has a lower limit of detection of 5 µmol/l for individual bile acid conjugates, and detection response is linear from 30–10 000 µmol/l. The lower limit for accurate quantification was therefore 30 µmol/l.

TABLE II Results (means) of preliminary 24 hour pH monitoring study in 52 patients and 20 control subjects

	No of episodes	No >5 min	Longest	%pH<4	No/hour	Min/reflux
Total:						
Control	9.9	0.9	10.6	1.7	0.5	3.2*
Patient	46.9	7.9	51.9	16.0	2.9	5.0*
Upright:						
Control	9.3	0.8	9.3	2.5	0.7	3.0*
Patient	36.2	5.1	30.0	15.5	2.4	3.6*
Supine:						
Control	0.6	0.1	2.4	0.2	0.1	1.0
Patient	10.7	2.8	39.0	15.8	1.6	8.8
Postprandial:						
Control	2.4	0.2	2.4	2.6	0.9	1.3
Patient	9.9	1.6	13.6	18.2	5.1	2.8

All comparisons significantly different except *p<0.05.

TABLE III Acid gastro-oesophageal reflux and oesophagitis (Medians (range))

% Time pH<4	0–I	II–III	IV	Barrett's
Total	8.3* (4.5–22.6)	18.1 (4.6–35.6)	17.4 (5.6–34.5)	20.1 (6.0–35.7)
Upright	9.1† (1.4–29.2)	15.4 (2.3–43.0)	15.2 (7.6–30.1)	16.4 (5.0–33.2)
Supine	3.7* (0–13.8)	13.6 (0–55.8)	17.0 (2.0–59.0)	17.1 (0–57.3)
Postprandial	7.4 (0.2–27.5)	15.3 (0–48.0)	21.4 (0–53.1)	17.9 (8.0–53.1)

*p<0.05 v II–III, IV, and Barrett's; †p<0.05 v IV upright.

Figure 1: Acid reflux from 24 hour pH monitoring in 52 patients subdivided according to grade of oesophagitis. Values are medians with interquartile range.

Unconjugated bile acids

To screen for the presence of free bile acids, an aliquot of each sample was subjected to thin-layer chromatography (TLC) after being deproteinated and resuspended in 20 ml of methanol. TLC was conducted in the solvent system 2,2,4-trimethyl-pentane, ethyl acetate, glacial ethyl acetic acid in the proportions 45:45:10 (by volume) after loading the samples (10 µl) onto 20 cm² Polygram Sil/uv254 (0.25 mm) plastic-backed plates (Camlab, Cambridge, UK). The samples were run against synthetic bile acids (Sigma Chemical, St Louis, USA) as standards. After chromatography, all bile acids were identified by spraying the plates with freshly prepared anisaldehyde reagent and heating in an oven at 100°C for five minutes to visualise the spots.

analyser (Technicon Instruments, Hants, UK). Specimens were incubated with benzoyl-arginine-p-nitroanilide (BAPNA) in 0.05 mol/l Tris buffer containing 0.02 mol/l calcium chloride at pH 8.2, and the rate of production of p-nitroaniline was monitored at 405 nm. Reaction rates were related to those of a series of standard trypsin solutions prepared from trypsin extracted from porcine pancreas (5000 U/mg).

STATISTICAL ANALYSIS

The Mann-Whitney U test was used for all between-group comparisons. Discriminant analysis used Wilks's method.¹⁸ Correlations were studied using Pearson's correlation test.

Results

Trypsin

Trypsin was assayed by the method of Erlanger,¹⁷ adapted to the Technicon RA1000

ACID REFLUX

All patients had an abnormal reflux fraction (>4.5%). All the separately measured para-

Figure 2: Proportion of the total acid reflux time occurring during upright, supine, and postprandial periods over 24 hours.

TABLE IV Ability of the parameters of acid exposure to predict the presence of oesophagitis using discriminant analysis (Wilks)

Parameter	Wilks's lambda	Significance
% Time pH<4 total	0.75670	p=0.0036
No/hour supine	0.63585	p=0.0014
No of episodes supine	0.49952	p=0.0001

63% of patients correctly classified with these three parameters; 59% classified without parameter time (or % time) pH <4 total.

TABLE V Oesophageal aspirates (Median (range))

Grade of oesophagitis	0-I	II-III	IV	Barrett's	Total
No of two hour periods studied	128	136	88	64	416
No (%) of aspirates	112 (88)	129 (95)	76 (86)	48 (75)	365 (88)
Volume of aspirate (ml)	16 (2-74)	16 (2-70)	25 (3-130)	25 (4-143)	17.5 (2-143)
Acid reflux/two hour period (min)	8* (1-85)	20 (1-120)	15 (1-100)	12.5 (1-120)	-
Pepsin concentration (µg/ml)	48 (0-530)	44 (0-500)	118† (0-564)	190† (0-553)	-
Bile acid concentration (µmol/l)	0 (0-15283)	0 (0-5226)	0 (0-5290)	0 (0-1092)	-

*p<0.003 v grades 0-I, II-III, and IV; †p<0.001 v grades 0-I and II-III.

meters of acid reflux, except upright oesophageal clearance (minutes/reflux episode), were higher in patients than controls in all periods (Table II). Patients with an endoscopically normal oesophagus (grades 0/I) had less reflux fraction than all other groups in both the 24 hour and 16 hour studies (p<0.05 and 0.003 respectively). No difference in reflux fraction (or any other reflux parameter) was seen between patients with erosive oesophagitis, stricture, or Barrett's oesophagus (Table III and Fig 1). The highest proportion of total reflux time occurred during the daytime in all groups (p<0.001) (Fig 2). For patients with oesophagitis the proportion of total reflux time was higher at night than in those with a normal oesophagus (p<0.01). In supine periods reflux fraction, number of reflux episodes, and number of episodes/hour were all

strongly correlated with the presence of oesophagitis (Table IV). In the Barrett's group reflux fraction was lower than grade 0-I when the patients were upright, but higher when supine (p=0.049 and 0.047 respectively). No differences were observed in patterns of reflux between those with erosive oesophagitis and complicated oesophagitis patients (stricture and Barrett's oesophagus).

PEPSIN

Sufficient aspirate for pepsin assay was obtained during 365 of 416 (88%) two hour periods, with a range of 75-95% between groups (Table V). The majority of periods where no aspirate was obtained were nocturnal. Aspirated volumes varied from 2 to 143 ml (median 17.5 ml). There was no difference in aspirated volume between each grade of oesophagitis. Pepsin was detected in 97% of specimens from periods containing acid reflux and in 11 specimens from periods without acid reflux. The highest concentrations of pepsin were found in patients with stricture and Barrett's oesophagus (p<0.001) (Table V) and were most evident during supine (nocturnal) periods (p<0.004) (Fig 3). There was no difference in pepsin concentration between the stricture or Barrett's groups, nor between grades 0-I and uncomplicated erosive oesophagitis patients (grades II/III).

SYMPTOM SCORES, VOLUME, REFLUX TIME, AND PEPSIN

Patients with Barrett's oesophagus had higher overall symptom scores than the other groups (p<0.01) (Fig 4). Those with erosive oesophagitis, stricture, and Barrett's oesophagus had higher total symptom scores than patients with reflux with a normal oesophagus (p<0.001). Pepsin concentration bore no relation to heart-

Figure 3: Pepsin concentrations in oesophageal aspirates from fasting, postprandial, upright, and supine periods. Values are medians.

Figure 4: Symptom scores for heartburn, regurgitation, and dysphagia for each grade of oesophagitis.

burn ($r=0.03$; $p=0.817$) and regurgitation ($r=0.23$; $p=0.1$). Total symptom scores correlated with pepsin concentration ($r=0.3$; $p<0.007$), but this was wholly contributed by a strong correlation between dysphagia and pepsin ($r=0.49$; $p<0.001$). Although minimum volumes were related to total symptom score, volumes of oesophageal aspirate were not related to scores for individual symptoms, even to those for regurgitation ($r=0.104$; $p=0.465$). A trend was seen when reflux time and total symptom score were correlated ($r=0.226$; $p=0.056$). Neither the duration of acid reflux nor the volume of aspirate for each period was related to pepsin concentration ($r=0.12$; $p=0.18$, and $r=0.014$; $p=0.48$ respectively). The duration of acid reflux during each period was not related to the volume of oesophageal aspirate ($r=0.083$; $p=0.28$).

BILE ACIDS

Ninety eight (27%) of the aspirates contained bile acids ($>30 \mu\text{mol/l}$), and 87 (89%) were obtained during periods in which acid reflux occurred. Thirteen (25%) patients had no detectable bile acids in their aspirates. There was no difference in the proportions of bile acid positive specimens between each of the subgroups according to grade of oesophagitis (range: 24–31%). Higher concentrations of bile acids were encountered during supine periods (Fig 5) ($p<0.001$). There was no difference in bile acid concentration between each grade of oesophagitis. Bile acid concentrations at levels known to be damaging to oesophageal mucosa in vivo ($>1 \text{ mmol/l}$) were found in only 2% of oesophageal aspirates and were equally distributed between each grade of oesophagitis (Fig 6). Free bile acids were not detected in any patient.

Figure 5: Conjugated bile acid concentrations in oesophageal aspirates from fasting, postprandial, upright, and supine periods. Figures in parentheses represent zero values.

Figure 6: Distribution of aspirates according to concentration of bile acids and grade of oesophagitis. Only 2% had concentrations >1 mmol/l.

TRYPSIN

Active trypsin was found in 17 of 365 oesophageal aspirates which were obtained from 13 patients. Only four specimens had trypsin concentrations >20 µg/ml. All of these specimens had a pH >4.6 , but only seven contained bile acids.

Discussion

The results show that oesophageal acid mucosal contact time is related to the presence but not the severity of oesophagitis in patients with gastro-oesophageal reflux. Patients with oesophagitis had a greater number of nocturnal reflux events than those without. Hence, although most acid reflux occurs during the daytime, nocturnal reflux seems to be more important in determining the presence of oesophagitis.

Active pepsin was found in almost all specimens from periods in which acid reflux was detected by pH monitoring, and since small amounts of pepsin (in an acid medium) can cause severe oesophagitis⁸ it is likely that active pepsin is an important cytopathic component of refluxed gastric juice. This assertion is further supported by the high nocturnal concentrations of pepsin found in patients with a stricture or Barrett's oesophagus, groups which were indistinguishable by acid reflux time alone. These high pepsin concentrations may be one explanation for the apparent sinister effects of nocturnal gastro-oesophageal reflux.

The pooled nature of the aspirates (in two hourly aliquots) does not permit a direct temporal analysis of the association between acid reflux (or 'alkaline' reflux) and other components of the refluxed gastric fluid. Nevertheless, the results indicate (not surprisingly) that pH monitoring does not give a measure of the likely concentration of pepsin in the oesophagus, since pepsin concentrations were not related to the duration of acid reflux for each two hour period. Neither were they related to the volume of

aspirate, suggesting that they may reflect gastric levels of pepsin.¹⁹

We found bile acids within the oesophagus of most of the patients with gastro-oesophageal reflux (mostly at night, but no less than 73% of aspirates contained no bile acid at all. The nocturnal tendency to increased gastric bile acid concentrations (probably due to increased duodenogastric reflux) has been previously reported,^{20,21} and bile acids have not been found in daytime oesophageal aspirates.²² All bile acids detected in this study were conjugated; the acid medium of the stomach does not permit bacterial proliferation which is necessary for deconjugation.²³ Conjugated bile acids are only injurious to the oesophagus in an acid medium, and most (89%) specimens containing them were obtained during periods in which acid gastro-oesophageal reflux was detected by pH monitoring. However, since only 2% of aspirates contained concentrations of bile acid sufficient to be damaging to the rabbit oesophagus *in vivo* (>1 mmol/l),²⁴ their contribution to the pathogenesis of oesophagitis seems likely to be minor. The fact that some patients with high bile acid concentrations in the oesophagus had only minimal or no mucosal damage, while many with low or undetectable bile acid concentrations had severe oesophagitis provides further evidence against a major pathogenic role for bile acids. Pepsin was present in 88% of specimens containing bile acids, but no evidence exists of an adverse synergistic effect on oesophageal mucosa when they are in combination.²⁵ Indeed, conjugated bile acids may even reduce the damaging effects of pepsin.²⁶ Trypsin (which is inactive at an acid pH) is highly damaging to oesophageal mucosa at an alkaline pH, and its injurious effects are considerably enhanced by the presence of unconjugated bile acids and conjugated bile acids in high concentration.^{26,27} No unconjugated bile acid was found in any patient in this study. Trypsin was seldom found in oesophageal aspirates and its low concentrations cast doubt

on any pathological importance. Because trypsin is inactivated by pepsin in an acid environment,²⁸ it may have been digested in those specimens in which ambient pH was acid. For pH >3.5 (when trypsin is stable in the presence of pepsin²⁹) it was only detectable in 17 of 301 specimens (6%). Even in those specimens containing high concentrations of conjugated bile acid and with a pH >6, implying appreciable duodenogastric reflux and thus an optimal situation for the discovery (and injurious activity) of active trypsin, no more than tiny amounts were found. It seems that most active trypsin cannot pass intact through an acid secreting stomach to reach the oesophagus.²⁹

Not surprisingly, patients with oesophagitis had more severe symptoms than those without, and patients with Barrett's oesophagus had the most severe symptoms. Patients with high pepsin concentrations in their oesophagus do not complain more of heartburn, but patients who complain of dysphagia are likely to have high oesophageal pepsin concentrations and will be likely to have a stricture or a Barrett's oesophagus. Interestingly, those with high volumes of aspirate from their oesophagus do not seem to have more regurgitation than those with low volume aspirates.

In 1935 Asher Winkelstein proposed that oesophagitis was the result of refluxed gastric contents, especially the digestive action of activated pepsin.¹ While a direct causal relation has not been established by this study, our results favour his concept of 'peptic oesophagitis' while finding no strong evidence of a pathogenic role for bile acids or trypsin.

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